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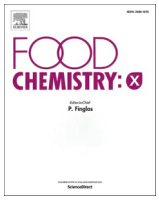

Molecular characterization and allergenicity assessment of different samples of Mung Bean

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ABSTRACT

Legumes represent a promising nutritional alternative source of proteins to meat and dairy products. Additionally, Novel Foods (Regulation EU 2015/2283) can help meet the rising protein demand. However, despite their benefits, emerging allergenicity risks must be considered. The aim of this work is the molecular characterization of the Novel Food Mung bean protein isolate for allergenicity prediction with High Resolution Mass Spectrometry analysis. The assessment of the allergenicity was evaluated in silico by comparing protein sequences of the Novel Food with other known legume allergens, using bioinformatic databases. The results highlighted similarity higher than 60 % of the protein structure of Mung bean with two known allergens of soybean and pea. Furthermore, enzymatic hydrolysis effects on allergenic potential was evaluated by immunoblotting analysis using sera of patients allergic to legumes. The protein hydrolysates obtained showed a high nutritional quality and a reduced allergenic potential, making them suitable for hypoallergenic food formulations.

1. Introduction

The UN's World Population Prospects 2022 estimated the total population at 8 billion in 2022; the latest projections suggest that the number will grow, reaching 10.4 billion by 2100 (DESA, 2022). One of the consequences of the population growth is the increasing demand for food. It is known that animal-based foods have a higher carbon and water footprint than plant-based foods (Curtain & Grafenauer, 2019).

Plant Based Diets (PBDs), represent a solution to reduce the environmental impact of agri-food systems, while improving human nutrition (Alcorta, Porta, Tárrega, Alvarez & Pilar Vaquero, 2021). Legumes represent an important aspect of new PBDs. They play a key role for food-system because they are a major source of sustainable plant-proteins and alternatives to animal products and production-system, since they are nitrogen-fixing species (Taylor, Marsh, Koppelman, Kabourek, Johnson & Baumert, 2021). Legumes are a valuable source of proteins, dietary fibres and phytochemicals and they are increasingly used in the formulation of new products due to their nutritional and health-promoting properties (Carbas, Machado, Pathania, Brites, Rosa & Barros, 2021). Nevertheless, their consumption could present some risks since certain compounds within them can significantly reduce their quality because they affect protein digestibility and nutrients bioavailability (Stagnari, Maggio, Galieni & Pisante, 2017). These molecules are referred to as antinutritional factors (ANFs), to which enzyme inhibitors, lectins, oligosaccharides, phenolic compounds, phytates and saponins belong (Wijaya, Zakaria, Syah & Prangdimutri, 2015; Verma, Kumar, Das & Dwivedi, 2013). Moreover, IgE binding proteins have been identified in the majority of legumes, thus leading to potential allergic reactions ranging from skin rashes to life-threatening conditions (Anvari, Miller, Yeh & Davis, 2019). Some legumes belong to the "Big 14", the 14 foods classified by the European Union (Regulation EU No 1169/2011) for which mandatory allergen labelling is required; other legumes, not classified as major allergens, may contain allergenic proteins, hence they may trigger allergic reactions in sensitive individuals (Anvari, Miller, Yeh & Davis, 2019). The majority of legume allergens can be categorized into four protein families and superfamilies: storage protein, which include the cupin superfamily (including 7S and 11S globulins) and prolamin superfamily (including 2S albumins and non-specific lipid transfer proteins); profilins; pathogenesis-related proteins (PR-10); defensins; oleosins (Cox, Eigenmann & Sicherer, 2021; Ballabio

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et al., 2010). Moreover, the increased consumption of legumes requires to consider the immune-cross-reactivities between legume seed proteins given by their sequence and molecular structure similarities. Several legume proteins share homology, but they are not all similarly allergenic (Ballabio et al., 2010).

Novel foods (NFs), represent a viable alternative pathway (Kumar et al., 2022; Russo, Songa, Marin, Balzaretti & Tedesco, 2020) to satisfy the needs of sustainability, food safety and security and make better use of available resources (Vågsholm, Arzoomand & Boqvist, 2020). NFs as alternative sources of protein can meet the challenges of a safe and nutritious food production, as they can meet the growth in demand for animal products (Pali-Schöll, Verhoeckx, Mafra, Bavaro, Clare Mills & Monaci, 2019). In the EU they are regulated by Regulation (EU) 2015/2283 of the European Parliament and Council on Novel foods (Russo, Songa, Marin, Balzaretti & Tedesco, 2020). Regarding the risk assessment it is important to consider the potential of NFs to cause immune-mediated adverse reactions. However, it is not possible to refer to a single property of NFs to predict their allergenicity (Pali-Schöll, Verhoeckx, Mafra, Bavaro, Clare Mills & Monaci, 2019). EFSA assumes by default that proteins present in a NF may have allergic potential; a novel protein may pose a risk due to *de novo* sensitisation or cross-reactivity (de Boer & Bast, 2018).

The Mung bean (*Vigna radiata*, L.) is a leguminous plant; it is rich in storage proteins, such as globulins, albumins and legumins; some of them have been already identified as allergens (Yi-Shen, Shuai & Fitzgerald, 2018). The allergens identified and classified in the WHO/IUIS schedule are Vig r1 pathogenesis-related protein, identified from its Bet v 1-homologous in Mung bean seeds (Mittag et al., 2005); Vig r2 (8S globulin), Vig r4 (seed albumin) and Vig r6 pathogenesis related protein, identified by purification and reverse-transcription PCR in Mung bean seedlings (Misra et al., 2011).

The Mung bean protein isolate (MBPI) extracted from the seeds was admitted by the European Commission as NF in the EU following the EFSA's risk assessment. It was proposed to be used as a food ingredient to be added to "protein products", whose proposed maximum use level is 200 g of isolate protein (dry matter (DM)) NF/kg food (EFSA, 2021). Since Regulation (EU) No 2022/673 only defines MBPI as a potential source of allergenic risk that consumption of this may trigger sensitisation, it becomes important to further investigate the allergenicity, cross-reactivity and methods to reduce the allergenic potential of this Novel Food. The IgE binding capacity and the allergenic potential can be reduced by the destruction or modification of allergen epitopes through physical, chemical or enzymatic processes (Kasera, Singh, Lavasa, Prasad & Arora, 2015). Among the various processes that can be applied to reduce the allergenic potential of food products, the enzymatic hydrolysis appears to be the most efficient, as can be seen from our recent work Calcinai et al., 2022: protein hydrolysates are characterised by a lower antigenic activity and can be used in the formulation of hypoallergenic formulas for foods. Moreover, enzymatic hydrolysis for reducing allergenic potential in soybean has been proven effective for the destruction of the major allergenic epitopes of soybean (Pi, Sun, Fu, Wu & Cheng, 2021).

The aim of this work was the characterization of the protein fractions, protein identification and allergenicity assessment of the MBPI Novel Food. Moreover, the aim of this work was also to produce the protein hydrolysates from MBPI and compare their allergenic potential to assess whether enzymatic hydrolysis is an efficient method for reducing the allergenicity of Novel Food.

2. Materials and methods

2.1. Mung bean protein isolate sample

The Mung bean protein isolate (MBPI) sample analysed in the present work has been supplied by the HI-FOOD SpA (Parma, Italy). The protein content (%) of the sample tested is 85 % as indicated in the product label.

2.2. SDS–PAGE (Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis)

The SDS–PAGE (Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis) analysis was performed on MBPI and enzymatic hydrolysates according to previous studies (Calcinai et al., 2022). To have a fixed quantity of protein to be injected in the gel, which was 0.03 mg of protein for MBPI sample and 0.05 mg of protein for enzymatic hydrolysates, the quantitation was carried out with the Quant-it protein assay kit using the Qubit™ Fluorimeter (Invitrogen by Thermo Fisher Scientific, Rodano, MI, Italy). 10 mg of each sample were diluted in 1 ml of dithiothreitol (DTT) 5 mM (ITW Reagents Division by PanReac AppliChem, Darmstadt, Germany), urea 4 M (VWR International S.r.l., MI, Italy) and ammonium bicarbonate 100 mM (NH_4HCO_3) (Sigma Aldrich, St. Louis, MO, USA) buffer. The mixtures were placed on a shaker at 200 rpm for 90 min.

The samples were then centrifuged (20.000 g, at 4 °C for 10 min) and the supernatants were filtered with syringe filters (0.45 μm pore size). One microliter of the sample solution was added to 199 μl of working solution (prepared with fluorophore and protein buffer solution 1:200). Then, the MBPI and the protein hydrolysates were separated on Criterion XT Bis-Tris Gel at 12 % (Biorad, Hercules, CA, USA) using the running buffer XT MES 20× (Biorad, Hercules, CA, USA).

2.3. In-gel tryptic digestion and high-resolution mass spectrometry by LTQ-Orbitrap

In-gel tryptic digestion analysis was performed on the most intense gel bands derived from SDS-PAGE analysis of MBPI sample, following standard procedures described in previous studies (Calcinai et al., 2022). For the analysis, the most intense bands in the samples were cut out from the gel. The peptides obtained from in-gel digestion were analyzed with High Resolution Mass Spectrometry (HR-MS) on the Thermo Scientific™ LTQ-ORBITRAP XL™ instrument. Afterwards LC-HR mass analysis, the protein identification was carried out by using the software PEAKS (Bioinformatics Solutions Inc) and the UniProt (www.uniprot.org) database. The Uniprot database cited was consulted from June to July 2023.

2.4. In silico analysis for allergenicity assessment

Allergenicity prediction of MBPI sample, an in silico analysis was evaluated by the combination of HR-MS results and proteomic web tools, as cited in the previous paragraph 2.3. Then, by Allermatch™ (www.allermatch.org) databases, the assessment of the allergenicity was evaluated in silico by comparing the amino acid sequence of the proteins in exam with other known allergenic proteins allergens, using the 80-amino-acid sliding window alignment (Codex Alimentarius Commission, 2008). Only proteins of known allergens with an identity percentage of more than 60 % and with experimental evidence at protein level were considered. After data filtering, protein sequences selected were analyzed with National Center for Biotechnology Information tool (NCBI) (https://www.ncbi.nlm.nih.gov/), in order to compare or align the amino acid sequence of protein of interest with the known one, using the Basic Local Alignment Search Algorithm (BLAST). All previous mentioned databases were consulted from June to July 2023.

2.5. Enzymatic hydrolysis of Mung bean protein isolate proteins

The enzymatic hydrolysis of proteins was performed in duplicate on MBPI, according to the procedure described in literature (Calcinai et al., 2022). The commercial proteases selected to perform the analysis were papain (PA), alcalase (AL) and flavourzyme (FL) (Sigma–Aldrich, St.

Louis, MO, USA). In order to perform the enzymatic reaction, 10 g of Mung bean isolate proteins, 50 ml of phosphate buffer at the appropriate pH for all the enzymes (pH 6.5 for papain, pH 7.5 for alcalase, and pH 8 for flavourzyme) and 1 % enzyme (w/w for papain, v/w for alcalase, v/w flavourzyme) have been mixed, placed in a water bath and kept under constant stirring (with magnetic stirrer) for 2 h, at 65 °C for the reaction performed with papain, 60 °C for the reaction performed with alcalase, and 50 °C for the reaction performed with flavourzyme. At the end of the reaction the enzymes have been inactivated by warming up at 90 °C for 10 min. The hydrolysates were then centrifuged at 3220g, at 4 °C for 30 min and the protein supernatant was separated from the pellet and lyophilized and stored at −20 °C.

2.6. Determination of protein content by Kjeldahl method

The analysis was carried out in duplicate on 200 mg of MBPI and the corresponding lyophilized enzymatic hydrolysate samples obtained after the procedure described in the previous 2.5 section. The protein content was determined with the Kjeldahl method, according to the AOAC International Official Methods of Analysis 2002 (AOAC, 2002). The final protein content of the sample was calculated by multiplying the determined nitrogen content by 5.6 (Mariotti, Tomé & Patureau Mirand, 2008) as the standard nitrogen-to-protein conversion factor for legumes. The protein content of MBPI determined by Kjeldahl was 75.2 % (±0.8) on dry matter.

2.7. Determination of the water content

The water content of the MBPI and enzymatic hydrolysate samples has been determined by loss on drying. All the analyses have been performed in duplicate. Each sample has been heated at 104 °C for 24 h (Tedeschi et al., 2022).

2.8. Determination of total amino acids

The total amino acids content was analysed using a previously published procedure (Prandi et al., 2021; Accardo, Leni, Tedeschi, Prandi & Sforza, 2022) with few modifications. For the determination of all amino acids except cysteine (Cys), methionine (Met) and tryptophan (Trp), 200 mg of dry MBPI and PA, AL and FL hydrolysate samples have been analysed (in duplicate) using an acid hydrolysis. Regarding Cys and Met, 50 mg of dry MBPI and PA, AL and FL hydrolysate samples have been determined by oxidation with performic acid. Two calibration line were made for the total and sulphured amino acids at 1.25 mM and 0.1 mM using Amino Acid Standard Mixture 2.5 mM (Thermo Fisher Scientific Inc). Then, the samples and the calibration curves were derivatized with an ACCQ Fluor derivatization kit (WATERS). Lastly, the content of tryptophan was analysed using a previously published procedure (Prandi et al., 2021) with some modifications. 100 mg of dry MBPI and PA, AL and FL hydrolysate samples have been analysed (in duplicate) using an alkaline hydrolysis. A solution of tryptophan (50 mg/100 ml) and α-methyl-tryptophan (50 mg/100 ml) was prepared as an internal standard. The samples have been directly injected in UPLC/ESI-MS using the Single Ion Recording acquisition mode. The analytical system is an Acquity UPLC coupled to a single quadrupole SQD detector (Waters, Milford, MA, USA), and the chromatographic column used is an Acquity BEH UPLC 300A, 150 × 2.1 mm with a C18 stationary phase (Waters, Milford, MA, USA). Details of the chromatographic and acquisition parameters are described in literature (Prandi et al., 2021).

2.9. Degree of hydrolysis (o-phtaldialdehyde analysis, OPA)

The degree of hydrolysis (DH) was determined on enzymatic hydrolysates following standard procedures described in literature (Calcinai et al., 2022). The DH was calculated as the ratio of free nitrogen groups after hydrolysis to total nitrogen groups, derived by the amount of protein calculated by Kjeldahl method. The free nitrogen groups are calculated from their reactivity of OPA/NAC. Total nitrogen groups correspond to the total moles of nitrogen in the system and are calculated from the ratio of total grams of protein to the average molecular mass of amino acids. The molar amount of free nitrogen groups was calculated against a standard calibration curve prepared with L-isoleucine.

2.10. Peptide analysis

Peptide analysis was performed on PA, AL and FL hydrolysates by VIon IMS QTof Mass Spectrometer (Waters, Milford, MA, USA), according to Accardo et al. (2022). Detection was achieved using a Vion IMS QTof Mass Spectrometer (Waters, Milford, MA, USA) using the same parameters in Accardo et al. (2022), exception for the acquisition time, which was 3.5–29.1 min. Data processing was performed using UNIFI software (Waters, Milford, MA, USA). The expected component list includes the following Uniprot protein accession numbers: Q198W5, A0A1S3W032, A0A1S3T8V4, A0A1S3VTQ0. The permitted variable amino acid modification is oxidation (M). Nonspecific digestion reagent, minimum sequence length: 3. To obtain the final list of peptides, data filtering was performed using the following criteria: high energy threshold, 75 counts; low energy threshold, 250 counts; max. number of peaks to keep per channel, 20,000; min. n° matched fragments, 2; min. % matched fragments, 20 %.

2.11. Peptides conservancy analysis

For this analysis, the focus was on those Mung bean proteins (Q198W5, A0A1S3W032, A0A1S3T8V4) that exhibited higher homology with the soybean Gly m 5 allergen (P0DO15, P11827, P25974) as demonstrated by in silico analysis (section 2.4).

Since several peptides of the four proteins of MBPI (Q198W5, A0A1S3W032, A0A1S3T8V4, A0A1S3VTQ0) (section 2.10) were identified from VIon IMS QTof Mass Spectrometer analysis, Protein Coverage Summarizer (PCS) software (http://omics.pnl.gov/software/ProteinCoverageSummarizer.php) was used to determine the coverage (%) and type of peptides for each enzymatic hydrolysate compared to soybean Gly m 5 protein sequences (P0DO15, P11827, P25974), identified by homology in the in silico analysis (section 2.4).

Then, for subsequent analysis, the shortest pieces of the same peptide, peptides with a length of less than 6 amino acids (Codex Alimentarius Commission, 2008) and repeats of the same peptide sequence were not considered.

The Immune Epitope Database (IEDB) (www.iedb.org) was used to search for the alignment of Mung bean selected peptide sequences within the known allergic epitopes of soybean (Gly m 5). Given the peptide sequences obtained from peptide analysis of PA, AL, and FL hydrolysates, our approach was to identify the best local alignments and determine the conservation of MB peptides into Gly m 5 soybean known allergenic epitopes. So, the selected peptides from each enzyme hydrolysate were directly entered into the IEDB repository, and local sequence alignments were searched against the known epitopes of the soybean allergen Gly m 5. The cited IEDB database was consulted in July 2023.

2.12. Allergenicity assessment

2.12.1. Immunoblotting assay

The human sera have been supplied by the Immunology Institute of the Santa Chiara Hospital of Pisa (Azienda Ospedaliero-Universitaria Pisana, Pisa, Italy). The experimental protocol was approved by the Ethical Committee of the Pisa University Hospital (Approval No 19008/2021). Informed consent was obtained from all subjects. The sera were collected from patients by ImmunoCap and Skin Prick tests, resulted in positive specific IgE to soybean, green pea and other type of legume, soybean and green pea. All the details of the human sera have been

reported in our previous study (Calcinai et al., 2022).

The immunoblotting experiments have been performed by testing 6 human sera, whose sensitization to soybean, green pea and other different legume species was known, shown in Supplementary Table 1 (ST1) of Supplementary Material. The tests were performed, on MBPI and enzymatic hydrolysates, following the procedure described in our previous work (Calcinai et al., 2022). Immunodetection has been carried out with the Bio-Rad ChemiDoc MP Imaging System (Calcinai et al., 2022).

2.13. Statistical analysis

Statistical analyses (one-way ANOVA, Tukey's test, *t*-test for equal variances) were performed using Microsoft Excel version 16.30 (Redmond, Washington, USA).

3. Results and discussion

3.1. Mung bean protein isolate characterization

The Mung bean protein isolate (MBPI) was provided by the company Hi-Food SpA. The protein profile of MBPI was analysed by SDS-PAGE gel electrophoresis, following the procedure described in section 2.2. Fig. 1, lane 1, shows the protein profile of MBPI, characterised by bands in the range between 20 and 100 kDa.

3.2. In silico allergenicity and cross-reactivity assessment

One of the several issues involved in the safety assessment of Novel Foods is the study and identification of new allergens. Proteomics tools may allow high accuracy of detection and quantification of food allergens in Novel Food, representing a method to study and validate allergen data (López-Pedrouso, Lorenzo, Gagaoua & Franco, 2020). Mass-spectrometry based proteomics has been carried out following a "bottom-up" approach, mainly as "shotgun" approach. These method starts from a chemical or enzymatic digestion to the detection and the fragmentation-based identification of the peptides allow the original protein to be identified by data searching and protein identification with bioinformatics tools. The targets are the specific peptides resulting from enzymatic digestion of protein extracts (Monaci, De Angelis, Montemurro & Pilolli, 2018). At the same time, in silico research to assess sequence homology and structural similarities with known allergens, provides an alternative for identifying food allergens within Novel Foods, due to the proteins sharing of high sequence homology, which could elicit allergic reactions in sensitized individuals. FAO and WHO set guidelines to establish cross-reactivity between expressed proteins and known allergens, which is based on the comparison of amino acid sequences: this occurs when (i) the percentage of identity (PID) of amino acid sequences is>35 % over a window of 80 amino acids or (ii) there is identity of at least six adjacent amino acids (Bianco, Ventura, Calvano, Losito & Cataldi, 2022). In silico approaches used bioinformatic tools to provide further information on cross-reactivity (Garino, Coïsson & Arlorio, 2016). The amino acid sequence homology comparison is performed using search bioinformatic engines, such as FASTA local alignment algorithm or the Basic Local Alignment Search Algorithm (BLAST) and the threshold value of 35 % identity over at least 80 amino acids (EFSA, 2022; EFSA, 2021).

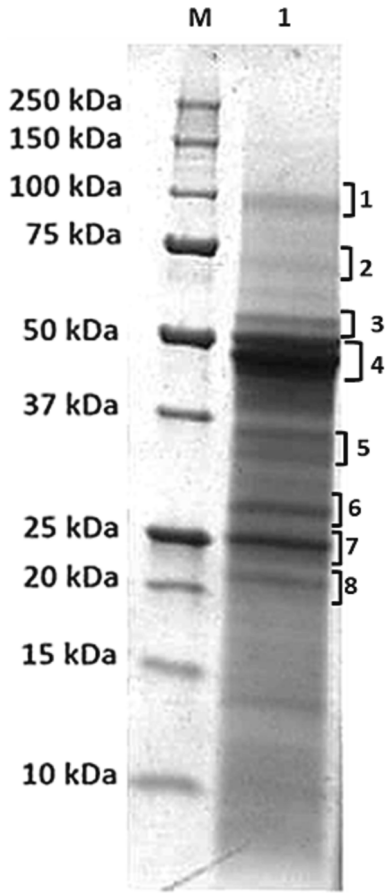

Fig. 1. SDS-PAGE of the MBPI: Marker (M); MBPI (lane 1).

3.3. In-gel tryptic digestion analysis

In this work, an in-gel tryptic digestion coupled with Orbitrap HR-MS analysis was performed on the most intense bands obtained by SDS-PAGE analysis, from band 1 to band 8 on MBPI (lane 1) (Fig. 1). Protein identification was performed by PEAKS and Uniprot database (paragraph 2.3). Positive hits for protein identification were arbitrarily set for all those proteins identified by the program with the higher percentage coverage (expressed as $-10\lg P$) > 50 and higher number of peptides ($-10\lg P$) > 30. The results of all the bands identified are presented in Table 1.

The most pronounced band (lane 1, band code 4, Fig. 1), has a molecular weight of approximately 50 kDa, attributed to the 8S globulin, which is the most prevalent in the MBPI.

This result is in line with previous reports in scientific literature, according to which the main seed proteins present in the Mung bean are the storage globulins of the vicilin type (8S approximately 90 %, 11S globulin and basic 7S globulin the remaining 10 %).

The 8S globulin vicilin-type fraction is composed of four types of

Table 1
Protein identification related to SDS-PAGE analysis (Fig. 1) and in-gel tryptic digestion coupled with HR-MS analysis (LTQ-Orbitrap).

Band code	Accession Number (Uniprot)	Coverage (%)	Number of peptides	Description
1	Q198W5	70	48	8S globulin α isoform
2	Q198W5	72	41	8S globulin α isoform
3	A0A1S3W032	58	45	β-conglycinin, α' chain
4	Q198W5	77	88	8S globulin α subunit
5	Q198W5	69	45	8S globulin α isoform
6	A0A1S3T8V4	71	36	β-conglycinin, β chain-like
7	Q198W5	68	64	8S globulin α isoform
8	A0A1S3VTQ0	32	39	Glycinin G4

subunits, 60 kDa (band code 3), 48 kDa (band code 4), 32 kDa (band code 5) and 26 kDa (band code 7), respectively, and presents three highly conserved isoforms (8Sα, 8Sα' and 8Sβ), which show high sequence identities (approximately 90 % identity); 8S globulins do not have disulphide bonds due to the lack of cysteine content. The 11S globulin legumin-type fraction was composed of two bands of 40 kDa and 20 kDa, and basic 7S globulin vicilin-type fraction was composed of 28 kDa and 16 kDa bands. Moreover, 8S globulin possesses a high level of homology (>60 %) and structural similarity with soybean β-conglycinin (7S globulin); this implies that 8S globulin in Mung bean could be characterised by properties similar to those of soybean 7S globulin (Liu, Liu, Yan, Cheng & Kang, 2015).

3.4. In silico homology assessment

Then, an in silico sequence homology evaluation was carried out between Mung bean proteins and known soybean allergenic proteins, since homologies between Mung bean proteins and those of soybean and other legumes, might lead to the phenomenon of cross-reactivity, thus being able to sensitise individuals and to induce allergic reactions in sensitive patients (EFSA, 2021).

Specifically, Vig r2 and Vig r4 are considered as clinically relevant allergenic proteins in Mung bean, which are able to induce strong IgE-mediated reactions (Misra et al., 2011). By performing HR-MS mass-spectrometric analysis, it was possible to identify Vig r2 as 8S globulin α-isoform precursor and β-isoform precursor, Vig r4 as seed albumin.

β- and α-isoform precursors of 8S globulin are major seed storage proteins of Mung bean belonging to the cupin superfamily; cupins are usually identified as major plant food allergens, and they include the 7S globulins of soybean (β-conglycinin, Gly m5) (Misra et al., 2011) and vicilins of garden pea (vicilin Pis s1 and convicilin Pis s2).

The in silico cross-reactivity assessment of the bands analysed in-gel tryptic digestion analysis is showed in Table 2.

For this analysis, only proteins with a % ID above 50 % were

Table 2
In silico allergenicity assessment of the MBPI.

Band code	Accession Number (UniProt)	Description	Protein (Allermatch™)	ID (%)	Species	Allergen	Accession Number (Uniprot)
1	Q198W5	8S globulin α isoform	β-conglycinin α-subunit 2	80.00	*Glycine max*	Gly m 5	P0DO15
			Vicilin	75.00	*Pisum sativum*	Pis s 1	Q702P1
			Convicilin	67.90	*Pisum sativum*	Pis s 2	Q9M3X6
2	Q198W5	8S globulin α isoform	β-conglycinin α-subunit 2	80.00	*Glycine max*	Gly m 5	P0DO15
			Vicilin	75.00	*Pisum sativum*	Pis s 1	Q702P1
			Convicilin	67.90	*Pisum sativum*	Pis s 2	Q9M3X6
3	A0A1S3W032	β-conglycinin, α' chain	β-conglycinin α'-subunit	80.50	*Glycine max*	Gly m 5	P11827
			Vicilin	68.80	*Pisum sativum*	Pis s 1	Q702P1
			Convicilin	64.20	*Pisum sativum*	Pis s 2	Q9M3X6
4	Q198W5	8S globulin α isoform	β-conglycinin α-subunit 2	80.00	*Glycine max*	Gly m 5	P0DO15
			Vicilin	75.00	*Pisum sativum*	Pis s 1	Q702P1
			Convicilin	67.90	*Pisum sativum*	Pis s 2	Q9M3X6
5	Q198W5	8S globulin α isoform	β-conglycinin α-subunit 2	80.00	*Glycine max*	Gly m 5	P0DO15
			Vicilin	75.00	*Pisum sativum*	Pis s 1	Q702P1
			Convicilin	67.90	*Pisum sativum*	Pis s 2	Q9M3X6
6	A0A1S3T8V4	β-conglycinin, β chain-like	β-conglycinin β-subunit 1	78.00	*Glycine max*	Gly m 5	P25974
			Vicilin	64.20	*Pisum sativum*	Pis s 1	Q702P1
7	Q198W5	8S globulin α isoform	β-conglycinin α-subunit 2	80.00	*Glycine max*	Gly m 5	P0DO15
			Vicilin	75.00	*Pisum sativum*	Pis s 1	Q702P1
			Convicilin	67.90	*Pisum sativum*	Pis s 2	Q9M3X6
8	A0A1S3VTQ0	Glycinin G4	Glycinin B3 Subunit	81.20	*Glycine max*	Gly m 6	P02858
			Glycinin B4 subunit	73.80	*Glycine max*	Gly m 6	P04347

primarily considered. The accession number of each MBPI band obtained by PEAKS tool, was entered into the UniProt database to obtain the amino acid sequence, which was entered into Allermatch, in order to predict and compare the potential allergenicity of MBPI proteins with other known allergens, as recommended by the Codex Alimentarius and the FAO/WHO Expert Consultation on Food Allergenicity (Codex Alimentarius Commission, 2008).

Indeed, it was found that the main similarity of the 8S globulin protein structure of MBPI is with soybean proteins, specifically 7S β-conglycinin. Gly m 5 and its isoforms (Gly m 5.0201 and Gly m 5.0101) are allergenic soybean β-conglycinins, while Gly m 6 is soybean 11S glycinin; the latter, comparing the amino acid sequence on NBCI BLAST tool, is similar about 75 % to the Mung bean glycinin G4, which is present at about 32 % coverage in all MBPI bands.

Then, the amino acid sequence of Mung bean 8S globulin and soybean β-conglycinins was compared using BLAST tool (https://www.ncbi.nlm.nih.gov/) and showed in Supplementary Fig. 1 (SF1) of Supplementary material. Thus, comparison of the protein amino acid sequence alignments of the two proteins, using the BLAST tool, confirmed a positive identity of 80 %.

It is also interesting to note that similarities were found between the MBPI proteins and those of the Garden pea (vicilins and convicilins). Currently, garden pea is not on the list of allergens whose presence in food requires mandatory labelling; however, several allergic proteins have been identified among its protein fractions, and some of these show similarities with those of the MBPI, such as vicilin Pis s 1 and convicilin Pis s 2 (Taylor, Marsh, Koppelman, Kabourek, Johnson & Baumert, 2021).

Due to such structural and amino acid sequence similarities between allergenic proteins of different species, IgE cross-reactivity between Mung bean, soybean and garden pea may occur in sensitised individuals.

For this reason, an in-vitro allergenicity and cross-reactivity assessment of MBPI was subsequently performed with sera of patients sensitive to different legume species, in particular soybean and pea.

3.5. Immunoblotting assay for allergenicity assessment

Immunoblotting assays were performed with 6 sera of patients allergic to soybean, green pea and other legumes in order to confirm the cross-reactivity discussed in previous paragraph.

Human sera used in the immunoblotting procedure were taken from patients characterised by the presence of IgE towards different legume species; detailed information was previously provided in section 2.12.1 and Table ST1 of Supplementary material.

The human sera A, B and D (Fig. 2, a, b and d) were characterised by similar immunoreactivity towards the "MB ISO" sample (second lane); in fact, the reactive bands were numbered correspondingly to the bands highlighted in MBPI SDS-PAGE gel (section 3.1). Bands that showed IgE-binding capacity were those of proteins with a MW of approximately 100 kDa (band 1), 60 kDa (band 3), 35 kDa (band 5), 30 kDa (band 6), 25 kDa (band 7) and 20 kDa (band 8). By comparing these results with what was obtained through the SDS-PAGE and in-gel tryptic digestion coupled with HR-MS analysis (sections 3.1 and 3.3), it was possible to identify bands 1, 5 and 7 as 8S globulins subunits; band 3 and 6 as β-conglycinin; band 8 as glycinin G4.

The reactivity of some of these proteins is also confirmed by results reported in the literature (Misra et al., 2011; Wijaya et al., 2015), where they are identified as Mung bean allergens: band 1, 5 and 7 probably corresponding to Vig r 2 Mung bean allergen; band 6 to Vig r 4 allergen; band 8 may match with Vig r 6 allergen. Therefore, these patients might develop allergic reactions triggered by proteins present in the Mung bean.

Reactivity towards the main proteins of the Mung Bean was also observed for D serum (Fig. 2d), for which previous clinical studies showed its immunoreactivity only towards green pea. This evidence was further confirmed on the basis of the previously performed in silico homology study (section 3.4), where cross-reactivity was found between Mung bean proteins and the two major allergens of green pea (Pis s 1 and Pis s 2).

On the other hand, the serum C shows reactive bands similar to those

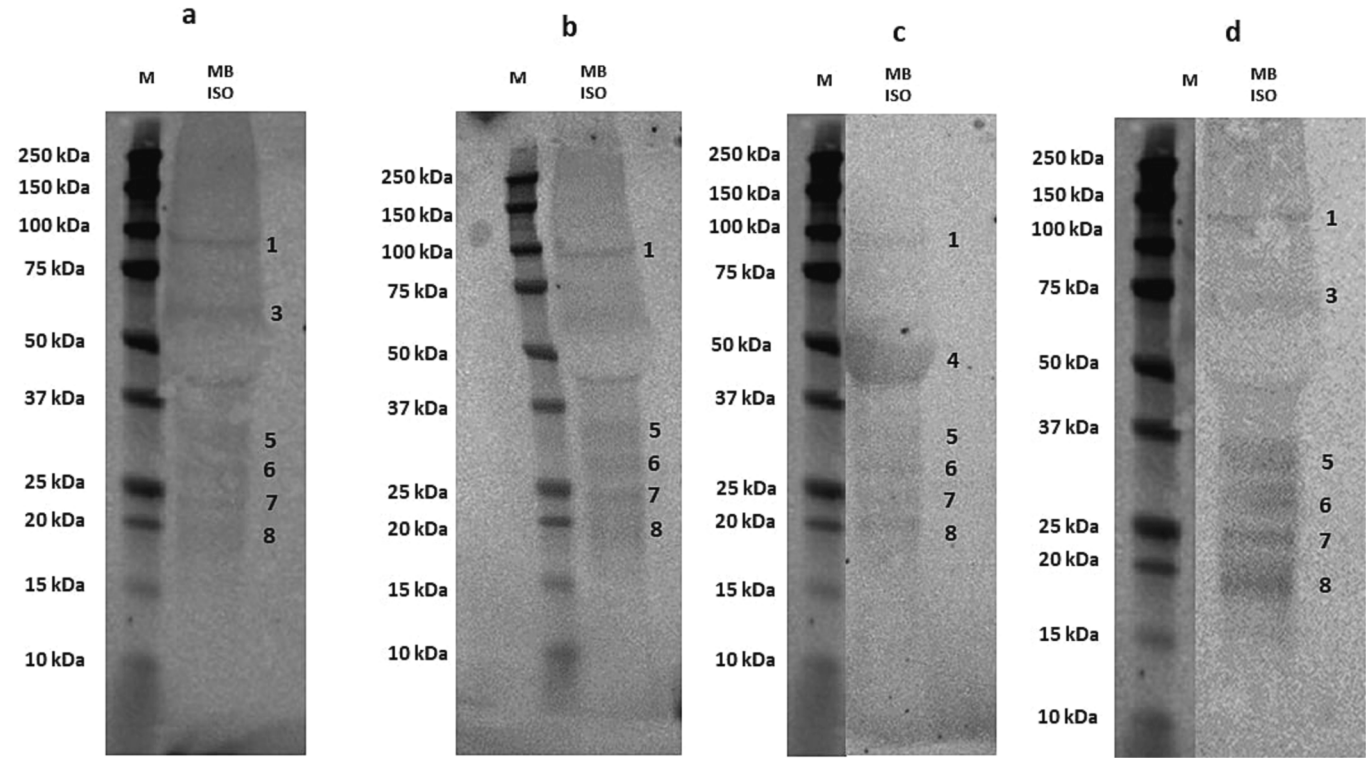

Fig. 2. Immunoblot of the MBPI samples. Performed on a) serum A; b) serum B; c) serum C; d) serum D. In all the immunoblotting images, left to right lanes: M: Marker; MB ISO: Mung bean isolate protein.

highlighted in Fig. 2a, b, and d, but also shows a reactive band with a MW of approximately 50 kDa (band 4).

Through the comparison of band 4 with the results of the SDS-PAGE and in-gel tryptic digestion coupled with HR-MS analyses (section 3.1 and 3.3), it was possible to identify it as 8S globulin α isoform. Studies in the literature report and confirm the allergenicity of this protein, which could be identified as Vig r 2 (MW of 52 kDa) (Misra et al., 2011; Wijaya et al., 2015).

Thus, the immunoblotting assays confirmed the homology between MBPI 8S globulin and soybean 7S globulin protein structure, due to the cross-reactivity in the sera tested who showed allergenicity to soybean.

3.6. Production of enzymatic hydrolysates from Mung Bean Protein Isolate

A further aim of this work is to study how the enzymatic hydrolysis can affect nutritional and allergenicity potentials of MBPI. The enzymatic reaction has been carried out using three commercial food grade proteases: papain (PA), alcalase (AL) and flavourzyme (FL). Papain and alcalase were selected as endo-proteases and flavourzyme as endo- and exo-protease.

The enzymatic hydrolysis reaction was carried out following the procedure described in section 2.5. In final samples, enzymes were inactivated with at 90 °C for 10 min; then, the supernatants were isolated by centrifugation and lyophilized. Firstly, the efficiency of the enzymatic reactions was evaluated by determining the protein reaction yield with Kjeldahl method; it was calculated as the ratio between the quantity of hydrolysed proteins and the total amount of proteins in the initial sample (in %). The results of the reaction yield differ considerably depending on the enzyme. The enzymatic hydrolysate with AL is characterised by the highest value of reaction yield (40 %), compared to PA hydrolysate (26 %), allowing to the highest efficiency. On the other hand, the FL hydrolysate shows the lowest value (16 %). This behaviour can be assumed to the different cleavage activity of enzymes. Endopeptidase, such as AL and PA, have a wide catalytic activity and cleaves peptide bonds within polypeptide chains. Flavourzyme is a mix of endoprotease and exopeptidase, but it is characterised by exopeptidase activity and mainly cuts amino acids from the end of polypeptide chains, leading to a different protein solubilization.

3.7. Evaluation of degree of hydrolysis by OPA/NAC analysis

The degree of hydrolysis is defined as the percentage of free amino groups in a protein hydrolysate and it gives an indication of the trend of the enzymatic reaction. DH is calculated as the ratio between the free nitrogen groups after the hydrolysis and the total nitrogen groups. To study the extent of the hydrolysis, DH % of the three protein hydrolysates has been assessed, following the procedure described in section 2.9. The results are shown in Supplementary Table 2 (ST2) of Supplementary material.

The DH % value of the enzyme hydrolysate obtained with the enzyme FL is the highest and is statistically significantly different from those of PA and AL.

The results obtained for the DH % of AL and PA hydrolysates are in line with those reported in literature (Liu et al., 2022). However, an explanation for the fact that FL leads to a higher DH (%) could be that this enzyme, compared to PA and AL, leads to the formation of more free amino acids and fewer peptides during hydrolysis. This data could be ascribed on the different way of action of the FL, which in principle exerts an exopeptidase and endopeptidase activity.

3.8. Protein characterization by SDS-PAGE electrophoresis analysis

SDS-PAGE gel electrophoresis has been used to analyse the protein profile of the MBPI and its three enzymatic hydrolysates (Fig. 3), following the procedure described in section 2.2.

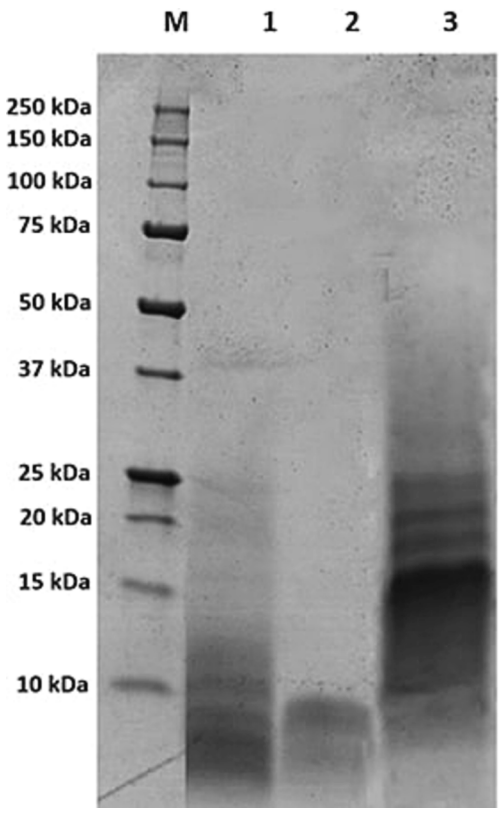

Fig. 3. SDS-PAGE of the MBPI enzymatic hydrolysates: Marker (M); enzymatic hydrolysate obtained with PA (lane 1); enzymatic hydrolysate obtained with AL (lane 2); enzymatic hydrolysate obtained with FL (lane 3).

The electrophoretic protein profile of the three hydrolysates shows a different proteolytic activity on the initial MBPI. This result is in line with what has been reported in the literature (Budseekoad, Takahashi Yupanqui, Alashi, Aluko & Youravong, 2019) and with the data of protein yield and DH (%) obtained in this work. Apparently, AL had a more effective proteolytic activity than PA: the hydrolysate shown in lane 2 exhibits weak bands and spots characterised by a low molecular weight of less than 10 kDa. In contrast, in the case of the PA hydrolysate (lane 1), light bands of higher molecular weight around 25 kDa, can still be detected. Regarding FL hydrolysate (lane 3), there are more low molecular weight bands. This thereby confirms the probable lower efficiency of this enzyme in the hydrolysis reaction compared to the other two enzymes. However, FL is able to hydrolyse proteins at higher molecular weights. The results obtained for the protein profile of MBPI and its hydrolysates through SDS-PAGE analysis are in accordance with those obtained for the reaction yield (%) and DH %, previously described. Furthermore, the results obtained for the hydrolysates show that the enzymatic hydrolysis can affect the protein profile of MBPI, depending on the different protease used.

3.9. Determination of the amino acid profile

In order to measure the quality and the nutritional value of proteins in different hydrolysates compared to the initial sample MBPI, as ingredients for food formulations, the total amino acid content was determined, according to the procedure previously described in section 2.8. The final result of the amino acids determination is summarised in Supplementary Table 3 (ST3) of Supplementary material.

According to the amino acid profile obtained in the present work, it is possible to notice that glutamic acid is the amino acid present in the highest quantities in the MBPI and in the three hydrolysates. High amounts are reported also for aspartic acid, leucine, lysine and

phenylalanine. Conversely, the contents of the sulphured amino acids (methionine and cysteine) and of tryptophan are the lowest. The results obtained are in line with those reported in the literature (Liu et al., 2022). The Mung bean has been identified as an excellent plant protein as it constitutes a rich source of AAs, especially the EAAs ones, compared with that of soybean, kidney bean and FAO/WHO reference protein (Du et al., 2018).

In conclusion, it can be stated that the enzymatic hydrolysates obtained with PA and AL retain a similar nutritional value to that reported for MBPI: the amino acid content after the enzymatic hydrolysis remained unchanged, and therefore the nutritional value was maintained at a high level.

3.10. LC-HR MS/MS analysis of enzymatic hydrolysates

Enzymatic hydrolysates samples were analysed by LC-HRMS/MS analysis with Vion IMS QTof Mass Spectrometer (Waters, Milford, MA, USA) and processed by UNIFI (Waters, Milford, MA, USA) software, according to the procedure describe in section 2.10. In Table 3 the list of identified peptides is reported, classified according to the different enzyme. Results shown a higher number of peptides for MBPI Alcalase, probably due to a greater amount of free amino acids content because of his better hydrolytic ability compared to Papain and Flavourzyme.

3.11. Peptides conservancy of enzymatic hydrolysates

Peptide local alignment of an unknown allergen compared with known allergenic epitopes can be performed using the IEDB tool resource (section 2.11). The IEDB is a comprehensive database that contains a vast collection of experimental data on immune epitopes, including allergenic epitopes. An epitope is a part of an antigen that is recognized by an antibody or antigen receptor.

IEDB provides access to information on known allergenic epitopes from various sources and allows the comparison of known allergenic epitopes similar to the sequence of query peptides to be analysed, helping the assessment of the potential allergenicity of unknown allergen.

Thus, the selected peptides (section 2.11) are screened and compared with known allergen Gly m 5 of soybean in order to determine if they could match with Gly m 5 known epitopes.

Table 3
List of identified peptides in each enzymatic hydrolysates.

Enzyme	Number Identified peptides	Protein source (Uniprot)	Description	Average peptide lenght
Papain	16	A0A1S3VTQ0	Glycinin G4	13
	67	Q198W5	8S globulin α isoform	7
	19	A0A1S3W032	β-conglycinin, α' chain	10
	17	A0A1S3T8V4	β-conglycinin, β chain-like	6
Alcalase	33	A0A1S3VTQ0	Glycinin G4	7
	145	Q198W5	8S globulin α isoform	7
	36	A0A1S3W032	β-conglycinin, α' chain	8
	78	A0A1S3T8V4	β-conglycinin, β chain-like	7
Flavourzyme	13	A0A1S3VTQ0	Glycinin G4	7
	84	Q198W5	8S globulin α isoform	9
	23	A0A1S3W032	β-conglycinin, α' chain	7
	52	A0A1S3T8V4	β-conglycinin, β chain-like	8

The degree of conservation of PA, AL and FL peptides within given set of protein sequences of soybean P0DO15, P11827 and P25974, which have a high homology with the 8S globulin of MB (section 3.4), was assessed with Protein Coverage Summarizer (PCS) software (http://omics.pnl.gov/software/ProteinCoverageSummarizer.php), as described in section 2.11. The results are shown in Supplementary Tables ST4, ST5 and ST6 of Supplementary material. The comparison of the peptides of the three enzymatic hydrolysates with the protein sequences of soybean P0DO15, P11827 and P25974, showed a coverage around 7 to 20 %. The peptides were selected using the parameters described in section 2.11 of material and methods. The selected peptides are underlined in grey colour in Tables ST4, ST5 and ST6. The minimum length of selected peptides was assessed ad 6 amino acids, according to the 2003 CODEX guideline on allergenicity (Codex Alimentarius Commission, 2008), where a query protein is potentially allergenic if: i) it has >35 % sequence identity over a window of 80 amino acids when compared with known allergens; ii) it has an identity of 6 to 8 contiguous amino acids with a known allergen.

Subsequently, by searching the IEDB database (https://www.iedb.org), we identified local MB peptide sequence alignments in the known epitopes of Gly m 5. The results are shown in Supplementary Tables ST7 of Supplementary material. By means of this analysis, some of these short local alignments were identified in different regions of the amino acid sequence of known epitopes of Gly m 5, for each type of hydrolysate. Thus, from these results, it would appear that enzymatic hydrolysis helps to mask hypothetical allergenic epitopes.

3.12. Protein allergenicity assessment of enzymatic hydrolysates

To assess the immunoreactivity of the protein hydrolysates, an immunoblotting analysis was performed on these samples, as described in section 2.12.1. Regarding the allergenicity assessment for the first four sera (Supplementary Fig. SF2 a, b, c and d of Supplementary material 1, third lane "MB PA", fourth lane "MB AL" and fifth lane "MB FL"), no bands were detected from the immunoblots in the enzymatic hydrolysates with PA, AL and FL, therefore it can be assumed that no IgE binding seems to occur at this level of sensitivity.

These results are consistent with the literature (Kasera, Singh, Lavasa, Prasad & Arora, 2015) and with our previous results obtained (Calcinai et al., 2022). They confirm the loss of conformational and linear IgE-binding epitopes following enzymatic hydrolysis, which resulted in a significant reduction in the allergenicity of the three hydrolysates. The results show how effective the combination of in silico and experimental analysis can be. Moreover, these findings are in line with the values obtained for DH % and SDS-PAGE analysis of the enzymatic hydrolysates showed in section 3.7 and 3.8, which showed an alteration and loss of protein structure, with the formation of small peptides and free amino acids. Therefore, the enzymatic hydrolysis process can be applied to reduce the allergenicity of the Mung bean proteins, as the peptides generated after the process can block antigen binding sites on the IgE, and thus inhibit further sensitization. This would constitute an advantage for the evaluation of hypoallergenic foods suitable for sensitive consumers.

Regarding the last two immunoblotting (Fig. 4e and f), these presented a slight immunoreactivity for all the bands in the first lane "MB ISO". As for the first four sera (a, b, c and d, Supplementary Fig. SF2), enzymatic hydrolysates with AL and FL not presented immunoreactive bands. Instead, even if the majority of immunoreactive bands of MBPI was reduced in the PA hydrolysate, the hydrolysis did not lead to a complete disappearance of immunoreactivity. Probably, proteolysis unmasked new epitopes in the protein band no. 7 (Fig. 4e and f, lane "MB PA"), identified through in-gel tryptic digestion analysis coupled with HR-MS (Table 1, section 3.3) as the 26 kDa subunit of 8S globulin; therefore, changing its IgE-binding capacity, and triggering a more intense reaction after the enzymatic treatment in both these last two

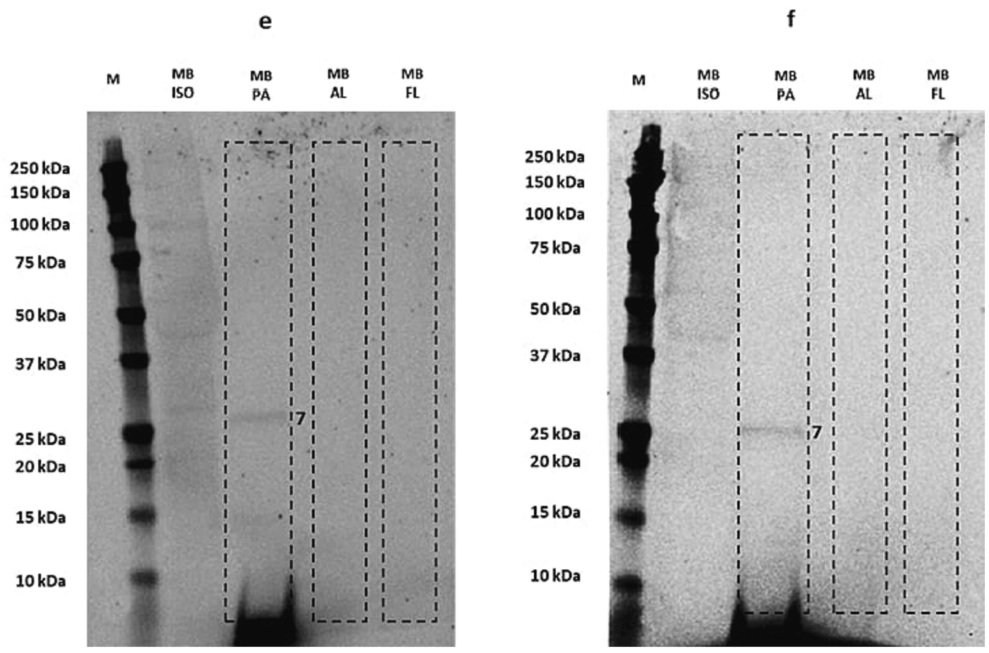

Fig. 4. Immunoblot of the MBPI samples. Performed on e) serum E; f) serum F. In both the immunoblotting images, left to right lanes: M: Marker; MB ISO: Mung bean isolate protein; MB PA: MB hydrolysate with Papain; MB AL: MB hydrolysate with Alcalase; MB FL: MB hydrolysate with Flavourzyme.

sera.

4. Conclusion

In conclusion, in silico analysis was performed to assess the protein quality and allergenic potential of Mung bean protein isolate and its enzymatic hydrolysates using papain, alcalase and flavourzyme. The in silico analysis revealed significant similarities between Mung bean 8S globulin (Vig r 2) and the major allergenic protein in soybean β-conglycinin (Gly m 5) and green pea proteins vicilin (Pis s 1) and convicilin (Pis s 2), suggesting potential IgE cross-reactivity. These findings demonstrate the effectiveness of in silico tools in characterizing allergens and the potential to use enzymatic hydrolysis to reduce allergenicity in MBPI Novel Food, making it suitable for hypoallergenic food products.

CRediT authorship contribution statement

Luisa Calcinai: Investigation, Writing – original draft. **Barbara Prandi:** Investigation. **Andrea Faccini:** Investigation. **Ilaria Puxeddu:** Conceptualization, Supervision. **Tullia Tedeschi:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100980.

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